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Propagation of the microsporidian parasite Edhazardia aedis in Aedes aegypti mosquitoes

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Journal of Visualized Experiments

Dear Editor,

We are happy to submit the revised version of our manuscript entitled “Propagation of the microsporidian parasite *Edhazardia aedis* in *Aedes aegypti* mosquitoes” for publication in JoVE as a part of the Methods Collection “The Study of Mosquito Biology.”

In this work, we detail the method for laboratory culture of the microsporidian parasite *Edhazardia aedis*. We demonstrate successful propagation of infection by quantifying *E. aedis* prevalence and parasite load in *A. aegypti* mosquitoes. *E. aedis* is an obligate parasite of *Aedes aegypti* and the parasite must be reared in live mosquitoes. Rearing of the parasite in mosquitoes requires controlled dosing of mosquitoes each generation and careful quantification of parasite loads. The general method has been published elsewhere, however, we feel that previous attempts to describe the rearing method have not been as detailed as would be necessary for a novice experimenter to be successful without substantial trial and error. We believe that the attached protocol will offer valuable clarification and detail to assist both novice and experienced researchers alike.

It is our hope that publishing this detailed protocol with high quality video and visual aids will increase accessibility to the practice of rearing *E. aedis* and allow more researchers to investigate the basic biology and applied potential of this system.

We have considered and addressed all suggestions from yourself and the reviewers, and we believe the manuscript is improved from its original version.

We appreciate your consideration of our revised work.

Best wishes,

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TITLE:

Propagation of the Microsporidian Parasite *Edhazardia aedis* in *Aedes aegypti* Mosquitoes

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KEYWORDS:

Edhazardia aedis, *Aedes aegypti*, microsporidia, parasite, mosquito, horizontal transmission, vertical transmission

SUMMARY:

A protocol to culture the microsporidian parasite *Edhazardia aedis*. The parasite is passaged from one generation of *Aedes aegypti* mosquitoes to the next via horizontal transfer at the larval stage followed by vertical transmission at the adult stage. Live sporoplasms survive long-term in infected eggs.

ABSTRACT:

Edhazardia aedis is a microsporidian parasite of *Aedes aegypti* mosquitoes, a disease vector that transmits multiple arboviruses which cause millions of disease cases each year. *E. aedis* causes mortality and reduced reproductive fitness in the mosquito vector and has been explored for its potential as a biocontrol agent. The protocol we present for culturing *E. aedis* is based on its natural infection cycle, which involves both horizontal and vertical transmission at different life stages of the mosquito host. *Ae. aegypti* mosquitoes are exposed to spores in the larval stage. These infected larvae then mature into adults and transmit the parasite vertically to their offspring. Infected offspring are then used as a source of spores for future horizontal transmission. Culturing *E. aedis* can be challenging to the uninitiated given the complexities of the parasite's life cycle, and this protocol provides detailed guidance and visual aids for clarification.

INTRODUCTION:

Aedes aegypti is the mosquito vector of multiple arboviruses (e.g., dengue, Zika, yellow fever) that together are estimated to account for hundreds of millions of disease cases each year and more than 30,000 deaths[1-2]. Treatment for diseases caused by these pathogens is limited to supportive care and it is likely that additional arboviruses will emerge in the future[3]. Control of the mosquito vector is therefore of primary importance, as it effectively prevents transmission of current and emerging pathogens[4]. Traditionally, vector control strategies primarily utilize chemical insecticides, but resistance to many commonly used insecticides has driven the demand for novel methods of vector control. One potential agent that has been explored for its biocontrol properties against *Ae. aegypti* is the parasite *Edhazardia aedis*[5-6].

E. aedis, first identified as *Nosema aedis* by Kudo in 1930, is a microsporidian parasite of *Ae. aegypti* mosquitoes[7]. The development and reproduction of *E. aedis* is relatively complex and its life cycle can proceed in multiple ways[7-9]. One common developmental cycle is described in depth in Becnel[7] and is utilized for laboratory propagation (**Figure 1**)[8]. Briefly, the cycle begins when *Ae. aegypti* eggs vertically infected with *E. aedis* hatch into infected larvae which develop uninucleate spores in the fat body, and usually die as larvae or pupae. Uninucleate spores released from dead larvae contaminate the habitat and are ingested by healthy *Ae. aegypti* larvae. These spores germinate primarily in the digestive tract, infecting digestive tissue of the exposed larvae, resulting in horizontal transmission. Horizontally infected larvae develop into adults (parental generation) where binucleate spores are formed. In the female, these binucleate spores invade the reproductive tract and their associated sporoplasm infects developing egg cells. These eggs then hatch into infected larvae (filial generation), resulting in vertical transmission of the parasite and continuation of the cycle as described above.

Multiple studies have investigated the potential of *E. aedis* for biocontrol. Infection with *E. aedis* has been demonstrated to result in diminished reproductive capacity of *Ae. aegypti* females[10]. Further, in a semi-field experiment, inundative release of *E. aedis* resulted in the total eradication of a test *Ae. aegypti* population kept within a screened enclosure[6]. While able to undergo some stages of development in a diverse set of mosquito species, *E. aedis* is only vertically transmitted in *Ae. aegypti*, indicating a high degree of host specificity[11-12]. Likewise, in a laboratory assessment of the potential environmental risk associated with *E. aedis*, the microsporidian parasite failed to infect non-target aquatic fauna, including predators that ingested *Ae. aegypti* larvae infected with *E. aedis*[13]. These results highlight the potential for *E. aedis* to be used in biological control strategies targeting natural *Ae. aegypti* populations.

Despite the fact that *E. aedis* shows promise for use in vector control, there are challenges to culturing and deploying it on a broad scale. *E. aedis* spores lose infectivity in less than one day at cold temperatures (i.e., 5 °C). Even at warmer temperatures (i.e., 25 °C), spores rapidly lose infectivity over the course of three weeks[14]. Additionally, *E. aedis* must be cultured in live *Ae. aegypti* mosquitoes and controlled dosing of healthy larval mosquitoes is necessary to ensure completion of the life cycle and to prevent collapse of the population used for culture[8]. The requirement of in vivo culturing presents a challenge; however, recent advances in mosquito mass rearing and robotics (e.g., Massaro et al.[15]) could allow for large-scale generation of *E. aedis*

spores. We anticipate that visualization of this methodology will increase accessibility to the *E. aedis* rearing protocol and allow more researchers to investigate the basic biology and applied potential of this system. We also anticipate that it will facilitate increased collaborations with engineers, roboticists, and the broader technology sector, which may serve to improve mass rearing of *E. aedis*.

PROTOCOL:

1. Day 0

1.1. Hatch *Ae. aegypti* eggs infected with *E. aedis* by placing in larval rearing tray with 1 L deionized (DI) water. Add 50 mg of fish food.

NOTE: At the time of publication, a laboratory strain of *E. aedis* is only available from laboratories actively researching the parasite, as *E. aedis* is not amenable to long-term storage and infected eggs are not currently stored in repositories. Researchers interested in working with *E. aedis* can contact the corresponding author to request infected eggs. Hatching large numbers of infected eggs is generally not necessary; ten *E. aedis* infected *Ae. aegypti* larvae are sufficient to dose ≥ 1000 healthy larvae.

1.2. For all parts of this protocol, house mosquitoes at the following conditions: 14 h/10 h light/ dark cycle, 27 °C temperature and 80% relative humidity.

2. Day 1

2.1. After hatching, reduce density of larvae to ~100 larvae per tray, making new trays as necessary (also with 1 L DI water).

2.2. Add a piece of dry cat food to each tray. Replenish food when depleted, but do not provide an excess of food. One piece of cat food (~200 mg) every three days is sufficient.

NOTE: Adjust food amount depending on the specific rearing conditions (i.e., reduce food if water becomes turbid or larvae are dying, increase food if larvae are severely delayed in development). Other feeding regimens and/or rearing conditions than those suggested here can be used but adjustments to timing of this standard protocol may be needed.

3. Days 4–5

3.1. When infected larvae are 3^{rd} – 4^{th} instars, hatch healthy/uninfected *Ae. aegypti* eggs in a new tray.

3.2. Rear at densities such that healthy *Ae. aegypti* reach 2^{nd} – 3^{rd} instar in 48–72 h. In our hands, this can be achieved using densities of 200–300 larvae per 1 L of water with *ad libitum* access to

food. Hatching batches of healthy eggs over multiple days can guarantee that larvae are at the correct stage when needed.

4. Days 7–8: Horizontal transmission

NOTE: Dosing of healthy larvae with *E. aedis* cannot be performed until uninucleate spores are at high numbers in infected larvae ($1 \times 10^4 – 1 \times 10^6$ per larva). This occurs late in the 4^{th} instar stage (**Figure 2**).

4.1. Harvest and quantify uninucleate spores.

4.1.1. Use a transfer pipette (one may have to cut the tip to a wider diameter) to move 10 infected larvae to a 1.5 mL microcentrifuge tube.

4.1.2. Remove breeding water with a 1 mL pipet and wash once by adding ~1 mL of clean DI water. Remove the wash water with a pipet, add 500 µL of clean DI water to the 10 larvae, and homogenize using a pestle and mechanical homogenizer.

4.1.3. Quantify spores using a hemocytometer at 400x magnification.

NOTE: Uninucleate spores can be identified by their distinct pyriform shape (i.e., pear shape; **Figure 2A**).

4.2. Dose healthy *Ae. aegypti* larvae with *E. aedis.*

4.2.1. Make fresh larval food slurry by mixing 1.2 g of liver powder, 0.8 g of brewer's yeast and 100 mL of water.

NOTE: Food does not need to be fresh if it is autoclaved and stored at 4 °C until use.

4.2.2. Transfer 100 $2^{nd} – 3^{rd}$ instar healthy *Ae. aegypti* larvae into 150 mL beakers or specimen cups.

4.2.3. Dose each beaker of 100 larvae with $5 \times 10^4 – 1 \times 10^5$ spores.

4.2.4. Add 2 mL of larval food slurry and DI water to a final volume of 100 mL.

4.3. After 12–24 h of exposure, transfer exposed larvae into rearing trays and rear to adulthood following a standard rearing protocol[16].

5. Monitoring and maintenance

5.1. Monitor dosed larvae for pupation and transfer pupae as they develop to an emergence cup in a cage. Sugar feed adults *ad libitum* (as per[16,17]). Eclosing adults will be infected by *E. aedis*.

5.2. Blood feed adults (as per[16,17]) and collect eggs. Vertical transmission of *E. aedis* occurs at this step.

NOTE: If additional blood meals are provided as soon as oviposition is complete, females can lay at least one additional clutch of eggs before adults suffer (often sudden) high levels of mortality. These *Ae. aegypti* eggs infected with *E. aedis* can be used to continue propagation starting with step 1 of this protocol. Eggs can be stored for 2 – 3 months under appropriate conditions[16].

5.3. Clean all materials that came in contact with *E. aedis* with 10% bleach and autoclaving (if possible) to prevent contamination.

REPRESENTATIVE RESULTS:

E. aedis infected *Ae. aegypti* Liverpool (LVP[1b12]) eggs were hatched as described in the protocol above. In the 4th instar stage, visual signs of infection could be observed, including white spore cysts throughout the fat bodies of infected larvae (an example of this phenotype is shown in **Figure 2B**). Uninucleate spores were harvested from 4th instar larvae by homogenizing 10 larvae in 500 µL DI water. These spores were pyriform (pear shaped) and readily visible at 400x (**Figure 2A**). Using a hemocytometer, a spore count of 4.05 x 10^3 spores/µL was calculated. One hundred healthy *Ae. aegypti* larvae were then horizontally infected with ~50,000 spores in 100 mL water for a final dose of ~500 spores/larva. Larvae were reared to adulthood (parental generation) and blood fed using defibrinated rabbit blood plus 1% (v/v) 100 mM adenosine triphosphate. Vertically infected eggs were collected (filial generation) and hatched to continue *E. aedis* propagation and to quantify infection success.

At seven days post-hatching, 25 filial generation larvae were transferred into individual 1.5 mL microcentrifuge tubes and washed once with DI water. Individual larvae were homogenized in 250 µL of DI water and infection status and *E. aedis* loads were assessed using a hemocytometer. Vertical infection rate of *E. aedis* in the filial generation was found to be 96% and the mean spore load of infected individuals at seven days post hatching was 3.31 x 10^5 (Range: 3.25 x 10^4 – 1.47 x 10^6; **Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: *E. aedis* propagation in *Ae. aegypti*. Propagation of *E. aedis* begins with hatching *E. aedis* infected eggs. Infected larvae are reared to 4th instar, *E. aedis* spores are isolated from those larvae, and the spores are used to orally infect healthy 2nd/3rd instar larvae reared from an uninfected clutch of eggs (horizontal transmission). These orally infected larvae are then reared to adulthood (parental generation) and lay eggs infected with *E. aedis* (vertical transmission). Infected eggs (filial generation) are then hatched to continue the infection cycle and parasite culture.

Figure 2: Visualization of *E. aedis* infection in *Ae. aegypti* mosquitoes. (**A**) *E. aedis* uninucleate pyriform spores. Ten *E. aedis* infected 4th instar larvae were homogenized in 500 µL of DI water

approximately seven days post hatching. 10 µL of the homogenate was loaded onto a hemocytometer and viewed at 400X. Red arrows indicate representative uninucleate *E. aedis* spores. (**B**) *E. aedis* infected 4th instar larvae develop distinctive white spore cysts throughout their fat body[18]. They also commonly have malformed and distended abdominal segments.

Figure 3: Culture protocol leads to effective *E. aedis* infection in filial generation: *Ae. aegypti* larvae (n = 25) from the filial generation were homogenized individually in 250 µL DI water and 10 µL of the homogenate was loaded onto a hemocytometer. Presence of uninucleate spores indicated a positive infection and spores were quantified for all positive samples. (**A**) Prevalence of infection among filial larvae. Grey corresponds to uninfected larvae, and black to infected. Numbers displayed on each segment give the absolute count of individuals in each group. (**B**) Spore load for each infected individual. Black dots represent the $\log_{10}$ transformed uninucleate spore count for each larva.

DISCUSSION:

We present here the method originally described in Hembree and Ryan[8] for rearing *E. aedis* microsporidia in *Ae. aegypti* mosquitoes. The strain of *E. aedis* used in this study was derived from the original field collection by Stephen Hembree in Thailand in 1979[19]. The method capitalizes on horizontal transmission, which naturally occurs in the transmission cycle of *E. aedis*[7], to propagate the parasite in a controlled manner. This method can be challenging to newcomers who are not familiar with spore appearance, symptoms of infection in larvae, or the coordination required to successfully complete the multi-stage rearing/dosing protocol. Our hope is that the visual aids that accompany this protocol will reduce barriers to entry for researchers who wish to culture *E. aedis.*

We propagated *E. aedis* in *Ae. aegypti* as described above and quantified the success of parasitism in the filial generation. Briefly, we hatched *E. aedis* infected *Ae. aegypti* eggs, reared them to 4th instar, and collected uninucleate *E. aedis* spores from the infected larvae. We then horizontally infected healthy larvae with these spores via oral ingestion, and reared the horizontally infected larvae to adulthood. We blood fed the infected adults (parental generation) and collected eggs (filial generation), which we hypothesized would be vertically infected with the *E. aedis* parasite. We hatched eggs from the filial generation and collected and homogenized a subset of the larvae when they were 4th instars. We quantified the percent of larvae that were infected with *E. aedis* and the total spore count in all infected individuals. We found that the vast majority (96%) of individuals were infected and the mean spore load of infected larvae was ~10^5. We conclude that our rearing protocol resulted in highly successful propagation of *E. aedis* in *Ae. aegypti* mosquitoes.

There are multiple aspects of this protocol that may be particularly challenging for the uninitiated user. We offer below some additional information that may be of assistance. For questions regarding general mosquito rearing, a complete guide to *Ae. aegypti* colony maintenance is beyond the scope of this protocol. However, many common questions can be addressed by resources from the Biodefense and Emerging Infections Research Resources Repository[16,17] including egg hatching, general dietary needs, housing and environmental conditions, and blood

feeding. Regarding the timeline of infection, larvae hatched from infected eggs do not show signs of infection until late in the 4th instar stage. Uninucleate spores appear rapidly, over the course of 1–2 days. Larvae may appear virtually uninfected at 6 days post-hatching but highly infected by day 7 or 8 post-hatching. Additionally, it can be challenging to visualize spores in homogenized samples because there are many other microbes present in whole mosquito homogenates, including other eukaryotic single-celled organisms (e.g., yeast) of a similar size as the *E. aedis* uninucleate spores. The distinctive shape of *E. aedis* spores (**Figure 2A**) is a highly reliable method for identification and will help differentiate *E. aedis* from other microbes in the homogenate. Though it is not necessary for identification or quantification, if spore purification is desired, it can be achieved via colloidal silica density gradient centrifugation which will allow for separation of *E. aedis* spores from other contaminating elements in the homogenate. This process is described in detail in Solter et al.[20].

Temperature and diet used in rearing practices commonly differ between laboratories, but variations will likely still yield successful parasite propagation. Minor differences in larval food type do not interfere with successful infection, though we did not explicitly test different food types in this protocol. The effect of temperature on infection has been tested and *E. aedis* infection was found to be robust at a wide range of temperatures[21]. Maximum spore production occurred at 30.8 °C but was still robust at rearing temperatures as low as 20 °C. Spore count was reduced dramatically at higher rearing temperatures (36 °C), therefore these temperatures should be avoided for this protocol.

Contamination is always a concern when working with parasites. *E. aedis* is a successful parasite of *Ae. aegypti* and must therefore be kept separate from uninfected laboratory colonies to prevent contamination. We recommend storage of infected mosquitoes in a separate incubator if possible. We also recommended that materials used for microsporidia work (e.g., larval trays, transfer pipets, cages, egg collection cups) are designated for microsporidia work and not used more broadly throughout the insectary. All rearing materials should be sterilized with 10% bleach after use and autoclaving can be used to supplement bleach sterilization.

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We wish to thank Spencer Blankenship for help with mosquito rearing. We also thank James N. Radl and M. Dominique Magistrado for helpful feedback on the manuscript.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

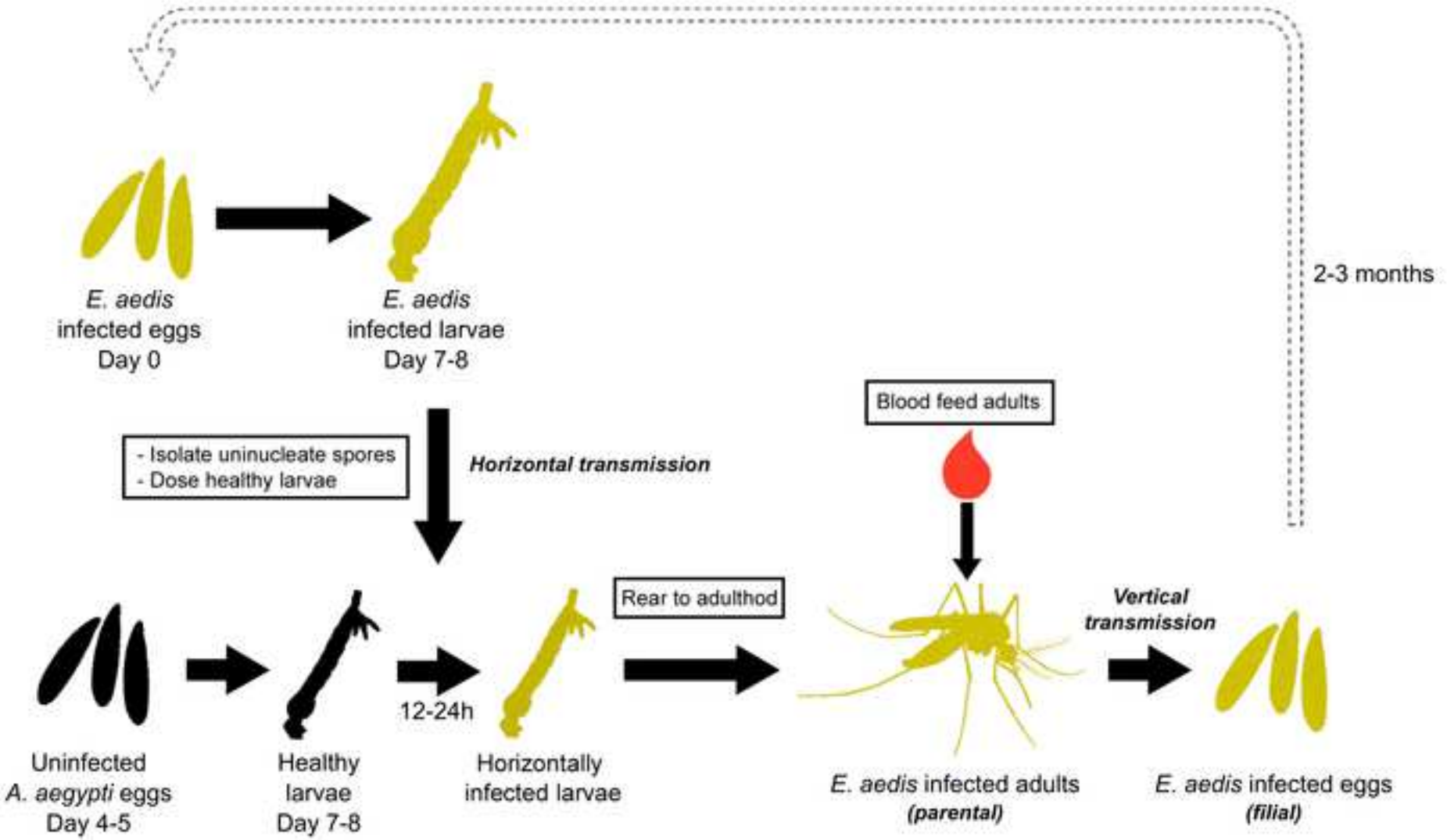

Figure 2

Click here to access/download;Figure;Figure2.psd

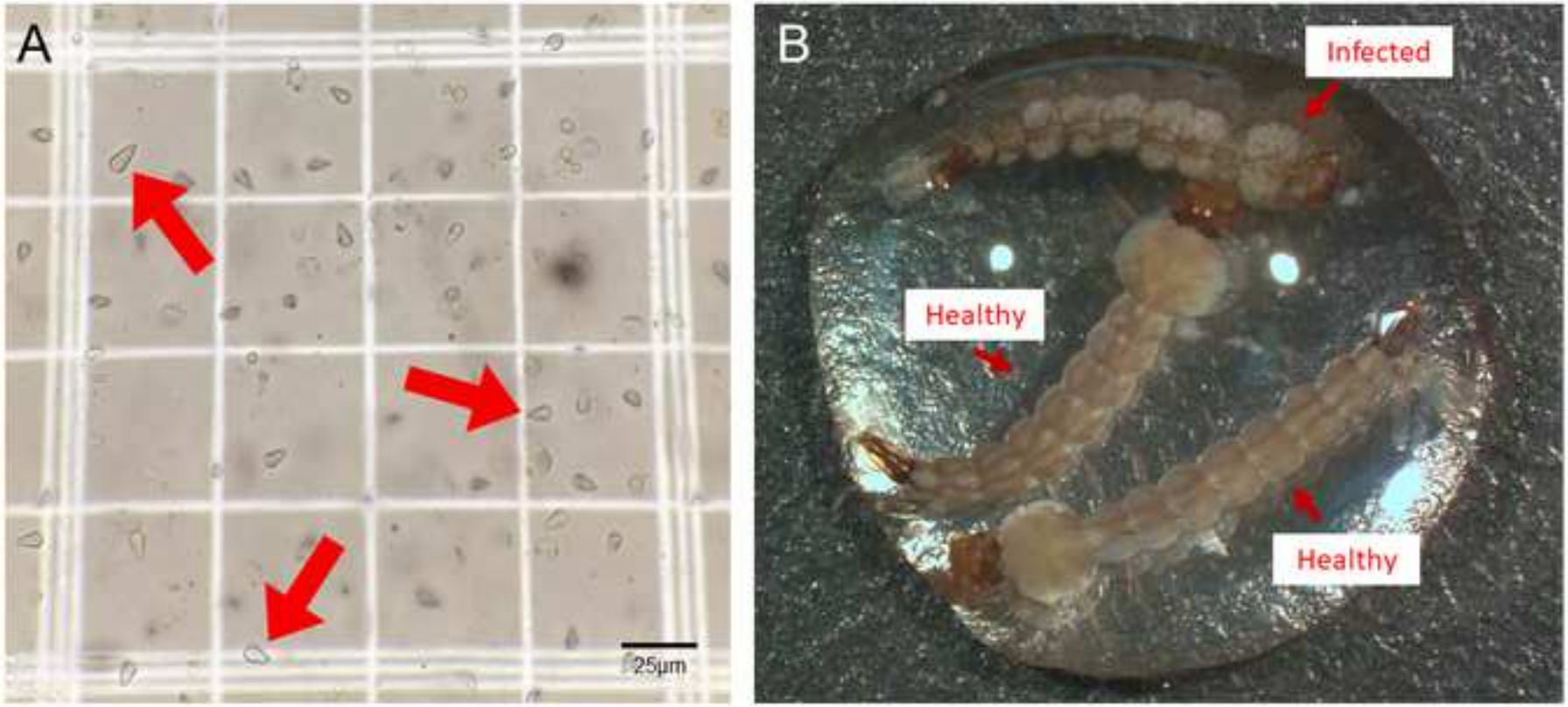

Figure 3

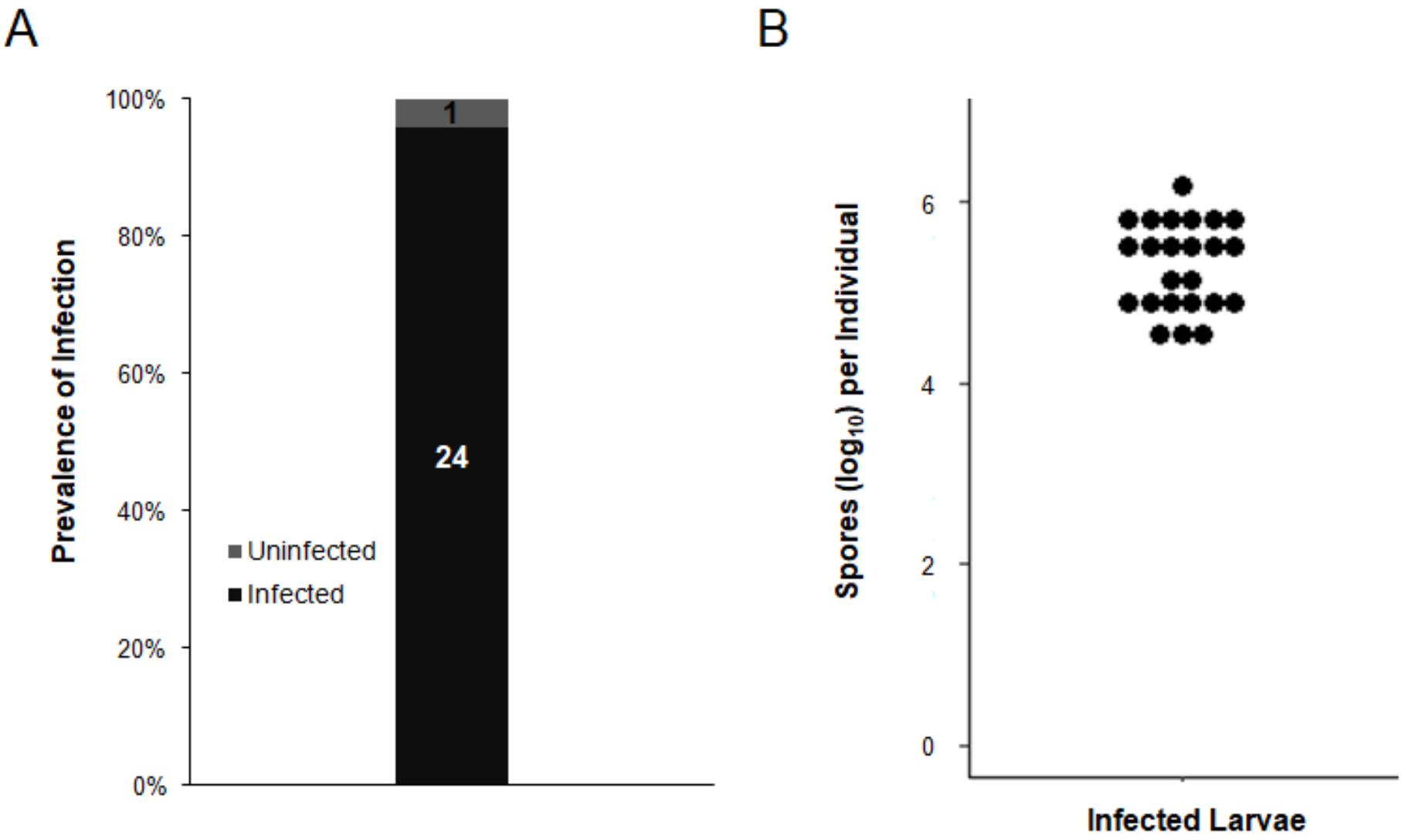

Table of Materials

Click here to access/download;Table of Materials;JOVE_Table_of_Materials-Revised_Final.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
120 mL Specimen cup	McKesson	911759	Inexpensive alternative to beaker
150 mL beakers	VWR	10754-950	For larval dosing
2 oz round glass bottle	VWR	10862-502	Bottle for 10% sucrose in adult cages
3 oz. emergence cup	Henry-Schein	1201502	For transfer of pupae to cage
Adult mosquito cages	Bioquip	1462 or 1450ASV	For adult housing
Autoclave			For sterilization
Bleach			For sterilization
Brewer's yeast	Solgar		For feeding larvae during dosing
Controlled rearing chamber	Tritech	DT2-MP-47L	Inexpensive small rearing chamber
Cotton roll	VWR	470161-446	Wick for sugar bottles
Defibrinated rabbit blood	Fisher	50863762	For blood feeding adults
Disodium ATP, crystalline	Sigma-Aldrich	A26209-5G	For blood feeding adults
Dry cat food	9Lives	Indoor Complete	For general larval rearing
Fish food flakes	TetraMin		For general larval rearing
Hemocytometer	Fisher	267110	For counting spores
Homogenizer/mixer motor	VWR	47747-370	For homogenizing infected larvae
Larval rearing trays	Sterillite	1961	Overall dimensions are 11" x 6 5/8" x 2 3/4"
Liver powder	NOW foods	2450	For feeding larvae during dosing
Pipette 1 - 10µL	VWR	89079-962	For larval dosing
Pipette 100 - 1000µL	VWR	89079-974	For food during larval dosing
Pipette tips 1 - 10µL	VWR	10017-042	For larval dosing
Pipette tips 100 - 1000µL	VWR	10017-048	For food during larval dosing
Plastic pestles	VWR	89093-446	For homogenizing infected larvae
Sucrose, crystalline	Life Technologies	15503022	For adult feeding
Transfer pipet	VWR	414004-033	For larval transfer, must trim ends

We wish to thank the editor and all five reviewers for their thoughtful and helpful comments on our manuscript. We have addressed each comment individually below in bold.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Thank you for this suggestion, we have done so.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We were unable to locate any issues of this nature in the manuscript. If there are specific details that you feel need to be added we would be happy to do so.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have reduced the number of paragraphs to 5 and ensured that they address only the subjects outlined here.

• Figures: Add scale bars to Fig 2 A,B.

We added scale bars to Figure 2A. Unfortunately, we do not have scale bar information for Figure 2B. In attempting to retrieve this information, we discovered that when the image was taken the scale bar did not register correctly. We have not been able to collect additional images due to COVID-19 related shutdowns. We appreciate the desire for a scale bar. However, we do not believe it is necessary to interpret Figure 2B nor do we reference any aspect of 2B in the manuscript that would require a scale bar to understand. Would you consider permitting an exception to your scale bar request in this case?

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

We have removed the brand name "Ludox" from the manuscript. We did not find any instances of trademark symbols in the table of reagent/materials. Please alert us if we have missed something or made a mistake.

• Table of Materials: Sort Alphabetically.

We have done this.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

This does not apply to our figures.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors describe the mass rearing protocol of a microsporidian parasite with the addition of clear representative results. This parasite may have important implications for the biocontrol of Aedes aegypti, the vector of many human diseases. The paper will help future studies with this microsporidia-mosquitoes system. Everything is well explained and detailed, the critical steps are highlighted. I wish I had a similar protocol some years ago, for, as the authors explain, the rearing of this parasite can be quite tricky. Overall, this is an excellent study, I have only few minor comments about some details.

Minor Concerns:
ll. 115-117. You could give an example of such densities to obtain the larvae for infetions 2-3 days later, or cite your references 16-17 in order for the reader to look it up.

This is a useful suggestion; we have incorporated it at lines 121-122.

ll. 152-153. More of a curiosity. The emerging adults are usually pretty weak. Adding some filter paper in the internal border of the cup helps a lot adult emergence and survival (well, personal observations). They can directly drink before to fly and have some sugar, and they have some extra grip to leave the cup. Did you try or are you doing it?

We are not using this approach, but this is very helpful information, thank you for sharing. Because we have not personally tested this approach, we are hesitant to include this in the manuscript, but we are grateful for the feedback.

ll. 176-177. I would stick only to pear shape, but I guess is quite personal.

We agree, and since "candy corn" is primarily found in the United States, we also have concerns that it may not be an appropriate reference for an international audience. We have removed it.

ll. 187. If you put the figure reference here, I would expect Figure 2A with the hemocytometer and not Figure 3.

This is a good point, we can see how it might be misleading. We have removed this reference and instead only reference Figure 3 in the next sentence, where we summarize the findings (Line 197).

Figure 1. This is likely the most important point/suggestion. This is a nice figure illustrating the protocol. However, I think it would be great if you could add labels with the day the precise steps occur. E.g. E. aedis infected eggs DAY 1, Uninfected A. aegypti eggs DAY 4-5, 2-3 MONTHS on the external dashed arrow and so on for all the steps. This would help to visually recall all the critical points and the timeline of the protocol.

This is a very helpful comment, we have added these values to the Figure 1, along with a few minor changes in the timeline to increase accuracy and clarification (changed day 1 to day 0, and changed day of spore isolation and horizontal infection to 7-8 so that the day range matches that of hatching healthy larvae).

Reviewer #2:
Manuscript Summary:
Authors provide protocol to propagate the microsporidian parasite Edhazardia aedis in Aedes aegypti mosquitoes including some filial infection data.

Major Concerns:
I suggest authors to add more data in infection rate of the horizontal transmission they performed and also rate of vertical transmission in subsequent generation.

We did not collect data on the rate of horizontal transmission, as our goal was to verify that the parasite was successfully vertically transmitted, and we feel that the data we included provide confirmation that our propagation technique is successful. We originally intended to collect a subsequent generation of infected filial larvae, however, we were unable to due to COVID-19 related shutdowns. Despite this lack of additional data, we believe we have shown clearly that the parasite is successfully propagated and our results are consistent with results previously published by ourselves and others who have used this technique successfully for many decades to propagate *E. aedis* (Hembree and Ryan, 1982; Becnel et al., 1989).

Why did they do filial infection for only 25 larvae?

Filial infection occurred during egg laying by the adults that were horizontally infected. Far more than 25 eggs were laid, and we hatched a clutch of eggs to assess filial infection rate. We did not record the exact number of eggs hatched. Our data represent a subset of the filial population.

Some part of the protocol is not clear such as
Line 95 why do they add fish food on the first dat?

We recommend fish food on the first day because it is more readily consumed by 1st instar larvae, which are very small.

Line 139 why dose it need fresh food for the larva?

To prevent microbial growth in the food. The food can also be autoclaved and stored in the refrigerator, but this is not necessary if the food is made fresh. We have added a note for clarification at Line 148.

Minor Concerns:
Some typing errors such scientific name should used full or short name consistancy.

Thank you for this useful feedback, we have made the recommended changes. We used the full form one time in the summary, abstract, and main body of the manuscript and then switched to using the short form consistently.

Reviewer #3:
Manuscript Summary:
This is an excellent manuscript that presents a protocol for propagating E. aedis in Aedes aegypti. The manuscript is excellent because it explains the importance of E. aedis, the challenges associated with culturing, and presents solutions. The manuscript is also clearly written, and the protocol takes a nice, step by step approach that anyone with the requisite reagents should be able to follow. In my opinion the manuscript can be accepted as is, but I present some minor suggestions that the authors should consider because in order to add further clarity to the manuscript.

Minor Concerns:
1. Line 94: Can the authors explain how investigators hoping to conduct research on E. aedis-A. aegypti can obtain the infected eggs?

This is an excellent point. Researchers would need to obtain infected eggs from laboratories currently researching *E. aedis*, as the parasites are not amenable to long-term storage and infected eggs are not currently stored in repositories. We have added text to this effect on lines 97-100.

2. Line 100: Is there an advantage to the recommended mosquito housing conditions that are recommended? (It is ok if it is simply that these conditions are known to work).

These conditions are simply what our laboratory uses and we know that under these conditions the protocol will yield the demonstrated results. There are a lot of variations around these conditions, however, that would work just as well. We altered the language in the protocol to indicate that this is what we did, and not that it is necessarily the best or only way to do it. (Lines 105-106)

3. Line 115: "rear at densities… in 48-72 hrs". Can some advice be given as to what that density is? I understand that it will change with temperature and food availability.

We have added this language at lines 121-122.

4. Line 206: Does the infected larva in the picture show the malformed phenotype? The segments look wider but I am not sure what to look for. Another thing I noticed is that the tracheal trunks that extend the length of the body are much darker in the infected larva. Is that phenotype specific to this infected larva or is it a common trait of all infected larvae.

The diagnostic for larval infection is the formation of white spore cysts throughout the fat body (see the white granular fat body in the infected larvae in Figure 2B. This is in contrast to the fat bodies of the healthy larvae, which lacks the "clumpy" appearance caused by cysts). We describe this in the figure legend as follows "*E. aedis* infected 4th instar larvae develop distinctive white spore cysts throughout their fat body", Lines 213-214. Since *E. aedis* does not cause systemic infection, secondary effects on physiologic systems (such as the tracheal trunks) would be speculative.

5. Line 253: Perhaps mention the advantages of purifying spores. The possibility of purification is mentioned but it does not appear to be necessary.

You are correct that it is not necessary for our protocol. Purification is desirable if the researcher wants to reduce the likelihood that they are horizontally transmitting other organisms or substances to the larvae or if they need pure spores in advance of DNA or RNA extraction. We wanted to mention the procedure since it is commonly used in this system. We have added clarifying language at lines 262-263.

6. Make sure that all the information in the Table of Materials fits in the same page. Right now the right-most column appears on a separate page.

Thank you for your attention to detail, we have fixed this.

Reviewer #4:

Manuscript Summary:

This is an excellent overview of the methods used to infect and maintain a microsporidian associated with Aedes aegypti. It will be very useful for any researcher hoping to propagate this organism in their laboratory.

Minor Concerns:

Although not necessary, I think it would be very useful to 'chart' the procedures on a sort of timeline, such that experiments can be better planned. There are quite a lot of steps that need to be done a certain number of days before another keys step so seeing this all visually would be super helpful.

We appreciate this thoughtful suggestion and have added approximate days to Figure 1 to help readers see the general timeline needed for propagation.

Reviewer #5:
Minor Concerns:
Line 94_ How the author obtained the initially Ae. aegypti eggs infected with E. aedis. Please provide the details.

This colony is a split from the original collection by Stephen Hembree in Thailand (Hembree, S.C., 1979. Preliminary Report of some mosquito pathogens from Thailand. *Mosquito News*, *39*(3), pp.575-582). Stephen Hembree sent infected eggs to Ed Hazard in Lake Charles in the early 1980s and the colony has been in continuous culture since. We have inserted information to this effect at Lines 227-228.

There are some repetition in the manuscript. It is advised to author to kindly thoroughly read the manuscript and correct it.

Thank you for this feedback, we have re-read the manuscript with an eye to reduce repetition. There is some repetition that is required for the JoVE protocol format, such as listing an overall heading of "4.2. Dose healthy *Ae. aegypti* larvae with *E. aedis."* And then indicating in subsequent steps how to specifically perform that task. Our understanding is that when formatted it will be more obvious that this is a heading, with subsequent steps falling under that heading.